3D Architecture and Replaceable Layers for Label-Free DNA Biochips

Yuksel Temiz, Sandro Carrara, Andrea Cavallini, Yusuf Leblebici,* and *Giovanni De Micheli*
Ecole Polytechnique Fédérale de Lausanne (EPFL) CH-1015 Lausanne (CH)
* Corresponding author: sandro.carrara@epfl.ch

Abstract - Recent advances in bio-sensing technologies have led to design of bio-sensor arrays for rapid identification and quantification of various biological agents such as drugs, gene expressions, proteins, cholesterol, fats, etc. Various dedicated sensing arrays are already available commercially to monitor some of these compounds in a sample. However, monitoring the simultaneous presence of multiple agents in a sample is still a challenging task. Multiple agents may often attach to the same probes on an array which makes it difficult to design a chip that can distinguish such agents (leading to low specificity). Thus, sophisticated algorithms for targets identification need to be implemented in biochip in order to maximize the number of distinguishable targets in the samples. The proposed algorithms are also required to introduce sophisticated signal processing and more intelligence on-chip. Dealing with these new processing and information technology demands constraints also require more innovative approaches towards hybrid integration technologies. To address such new demands, we discuss in this paper an innovative 3D-integrated bio-chips especially dedicated to label-free DNA detection.

I. INTRODUCTION

DNA microarrays are the most commonly used biosensors for gene expression measurements. They work on the principle of identifying mRNAs in a sample using complementary cDNA strands as probes. In addition to the quantification of gene expression, microarrays can also be used for identifying the presence or the absence of an organism in a sample. In such an application, a set of oligonucleotides (synthetic single strand DNA) can be used as probes that hybridize to the genome of the organism under investigation. The same principle can be extended to the identification of a certain set of viruses in a sample. In that case, the probes used are specific to the genome of each virus in the sample. This would work if the genomes of the viruses are very different from each other so that the designed probes can be unique to each virus. But very often biological applications, such as monitoring of water pollution, would require identification of viruses that are closely related or have similar genome. In such situations, it becomes difficult to find unique probes for each virus. To monitor multiple targets in a sample, a non-unique probe design method has been proposed in the past [1, 2], where the designed probes may hybridize to multiple targets (or viruses) such that it is still possible to differentiate between any two viruses in the sample. A possible solution of the generalized formulation for distinguishing the simultaneous presence of DNA–targets in a sample has been given in [3]. This fact demands for new, advanced and innovative systems able to address more intelligence on board. This can require large amounts of memory and computation, a serious drawback for portable and real-time applications. High level of programmability and flexibility with 3-D Chip hardware fabricated by using 0.7-µm CMOS technology has been demonstrated for individual classifiers [4]. Thus, 3-D chip architectures are suitable for implementing more intelligence in fully-electronic and portable DNA chip. Complex fully electronic DNA chip should also be reusable for economical reasons. Reusable chip concept can be addressed by developing a replaceable bio-layer on top of the 3D-stack chip structure. Aim of this paper is to present an innovative solution for 3D-DNA-Biochip with replaceable top layer and to show preliminary results in developing such a system.

II. 3D CHIP WITH REPLACEABLE BIOLAYER

For decades, the continuing demand for faster and smaller integrated circuits (ICs) has led to aggressive downscaling in transistor sizes. As a result, today's state-of-the-art IC technology has offered fascinating levels of performance and functionality, while introducing new barriers and challenges for further downscaling. As conventional planar IC designs have almost reached their limits, the three-dimensional integrated circuit (3D-IC) technology offers new possibilities to continue the trend predicted by Moore's Law [5]. This emerging technology enables the integration of multiple layers with vertical interconnections, providing potential performance improvements even in the absence of continued device scaling [6]. Today, there has already been an extensive research on 3D memories, FPGAs, and multiprocessors, where each individual chip in the stack has identical or similar properties. Besides this homogenous integration, the 3D-IC technology also offers novel opportunities for the realization of systems comprising heterogeneous layers, such as RF, analog, digital, MEMS, etc. We believe that this technology also holds great promise for biosensor arrays, which is totally a new application area for this technology and still conceptual. Figure 1 shows the illustration of a 3D-integrated biosensor system composed of heterogeneous chips [7]. In this system, it is aimed to improve overall performance by employing different fabrication technologies for each particular function, such as custom micro-fabrication for the bio-layer, specific technologies enabling low-noise operation for analog electronics, and high speed/density CMOS technologies for digital circuits and memories.

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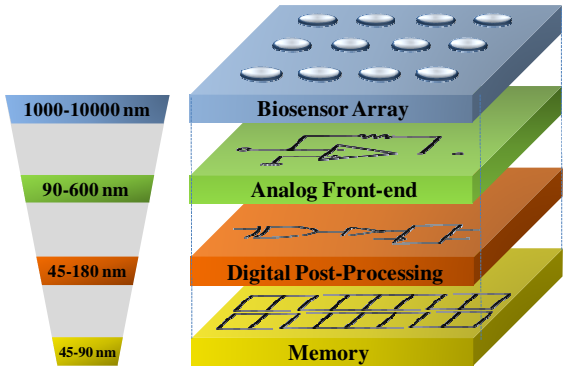


Figure 1: 3D integration of heterogeneous bio-sensor systems on chip (reprinted from [7]).

Although 3D integration supports realizing complete biosensor systems having superior performance and higher array densities, passive electronic biosensors still hold the advantage in terms of disposability. For instance, in active-electronic (monolithically integrated) biosensors, biologically active area has to be cleaned after each measurement. This cleaning process usually damages the electrodes and sensitive electronics, causing decrease in the sensitivity or even breakdown of the system. Moreover, especially in the clinical diagnostic applications, it is preferred to use each sensor once to eliminate the contamination, such as contamination due to blood samples. One way is to dispose the complete system every time; however, this is not economical for such complex systems comprising CMOS electronics. Therefore, in this study, it is aimed to develop bio-layers which can be attached on top of a 3D CMOS stack and replaced after each measurement so that the overall system can be used many times without the necessity of any cleaning process. We believe that this approach will combine the advantages of both active-electronic and passive-electronic biosensors in a single system. Figure 2 shows the simplified illustration of the 3D integrated biosensor with detachable biolayer. In the proposed approach, biolayer is temporarily connected to the analog interface circuit through vertical vias having contact surfaces well-matched to the (111)-Si planes for better alignment. Vertical interconnections allow the fabrication of high-density bioarrays since they solve the horizontal signal routing problem of disposable biosensors. In addition, since the interface electronics is in the direct neighborhood with the bioarray, weak sensor signals are amplified by being minimally affected by the parasitics, leading to considerably improvement in the sensitivity and the signal integrity. Moreover, analog outputs are converted to digital signals through on-chip ADC converters and processed by dedicated digital electronics present in the 3D CMOS stack. Hence, the signal processing required to solve complex algorithms for target identification can be accomplished on the system. This technology also allows employing different measurement techniques (cyclic voltammetry, capacitance

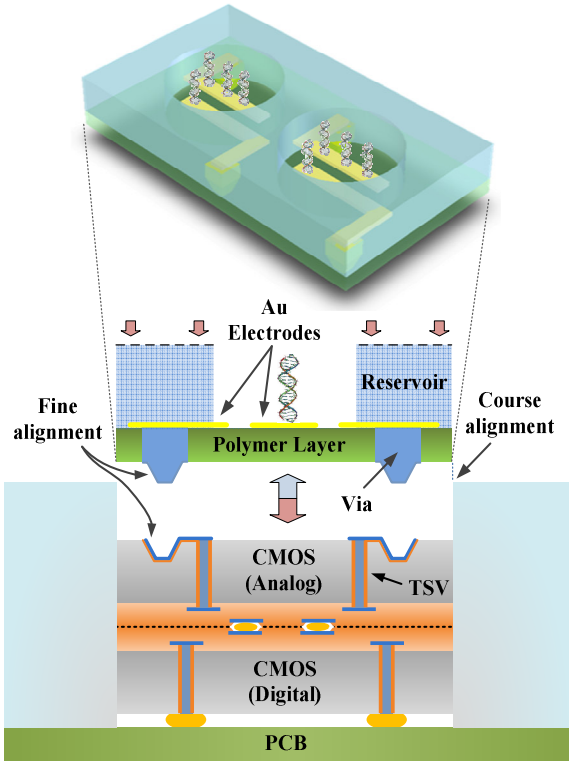


Figure 2: A simplified illustration of the proposed 3D integrated biosensor with detachable bio-layer.

measurement, etc.) by simply replacing the biolayer and configuring the analog front-end accordingly.

The fabrication of the proposed system involves through-silicon-via (TSV) technology [5] for the CMOS stack and custom micro-fabrication for the biolayer. Figure 3 shows the basic steps of the process flow for replaceable biolayer fabrication. First, via regions are etched by KOH on a (100)-Si wafer having a thin oxide layer on top. After a thermal oxidation step, the surface is coated with a polyimide (PI) layer, which will act as a substrate for the electrodes. The PI is then anisotropically etched and the openings are filled with Cu electroplating by using a Cr/Au seed layer. After polishing the surface with CMP, Au lift-off process is employed to form sensing electrodes on the PI surface. A separate Si wafer is etched with DRIE process and bonded to PI surface by adhesive bonding. This layer both acts as reservoir and gives the structure enough thickness for handling. Finally, sacrificial oxide layer is etched and the biolayer is released from the silicon substrate. After the fabrication of the biolayer with vertical interconnections, DNA probes can be immobilized on Au electrodes. However, before using the novel biolayers, tests on probes immobilization for reliable label-free DNA detection were verified with passive-electronic (disposable) biosensors fabricated on glass substrates.

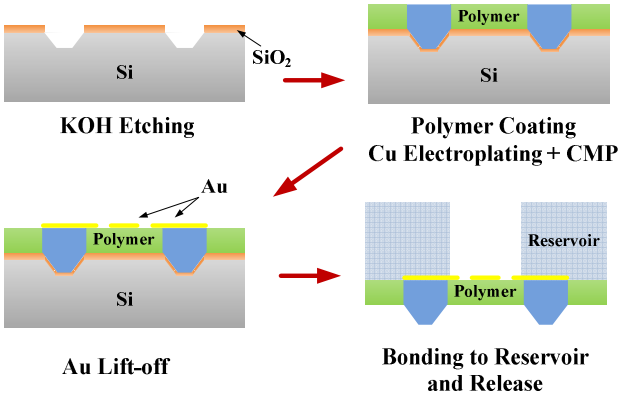


Figure 3: Basic steps of the process flow for replaceable bio-layer fabrication.

III. DISPOSABLE BIOCHIP FABRICATION

Each chip was drawn with 32 square electrodes of 200µm each, equally divided in 4 different areas. In each area, the 8 electrodes were divided in 4 pairs. Two different layouts for the electrode disposition were designed:

- Layout 1 – standard (figure 4): Vertical distance between electrodes: 20µm; horizontal distance: 860µm. Only 4 different measurements in each area are allowed with this configuration.

- Layout 2 – hybrid (figure 5): Vertical distance between electrodes: 20µm. In this configuration the inner couples are at a horizontal distance of 20µm. The gap between inner and outer pairs is 1300µm. This layout gives the possibility to make 2 additional measures in each area (inner couples can be also measured in horizontal direction). These two measures give a better estimation of capacitance of one area, and increase the durability of the chip (if a path is broken it is still possible to have a sufficient number of measurements).

Each electrode has a gold path of 20µm width for the connection to contact pads. Contact pads have dimensions of 1x1 mm. Like the electrodes, every pad is identified by a number and a letter. The layout of the pads was suitable for the chip docking in a hand-made probe station built for a previous chip used in our laboratory.

Chips were produced in 100 mm – diameter wafers. A total of 8 wafers were processed, each containing 3 biochips with hybrid circuit layout and 1 with the standard one. Every wafer also contains 32 additional test spots for further measurements. The whole wafer was covered with an insulating layer of SiO_2 that has to be removed above the electrodes and contact pads, in order to expose the gold surface. A second mask (opening mask) was then designed above the metal mask, because it was used to create "holes" on the SiO_2 surface. A positive aperture (which means bigger than the metal mask elements) was drawn for electrodes: each has an opening of 220µm width, thus a gap of 10µm is created all around the gold electrode (figure 6) because the space inside the electrode couple is 20µm. The opening is sufficient to create a single "hole" in which the pairs are hosted. For contact pads, opening has a negative aperture (it is confined inside the metal mask elements): its width is 980µm, thus the edge of the contact pads (10µm width) is protected with SiO_2. Finally, standard lift-off process was used to pattern the gold electrodes onto the glass substrate. To improve adhesion between the substrate and the electrodes first a 20nm layer of Chromium was deposited, followed by a 200nm layer of Gold using thermal evaporation. The entire surface was covered by a thick (10µm) layer of SU8 photoresist that acts as a passivation layer. Individual sensor spots were exposed by developing SU8. The chip contains a total of 32 square electrode arranged in groups of four electrode pairs. Figure 6 shows one chip spot in detail. The electrode square side is 200µm and the electrode's separation is 20µm.

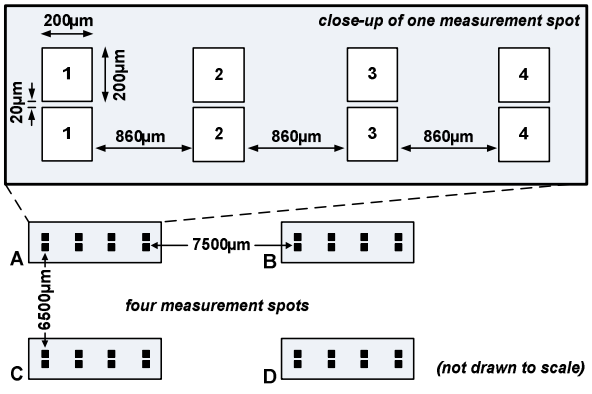


Figure 4: Schematic view of the standard layout with quotes.

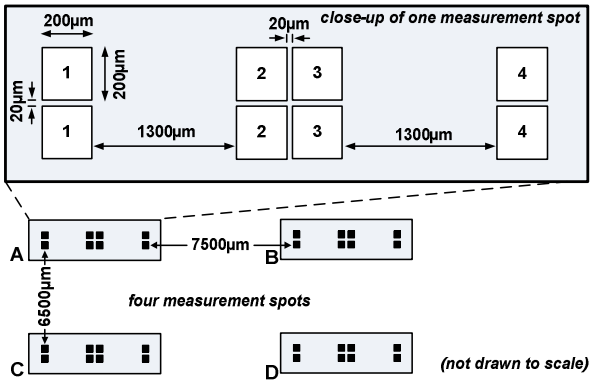


Figure 5: Schematic view of the hybrid layout with quotes.

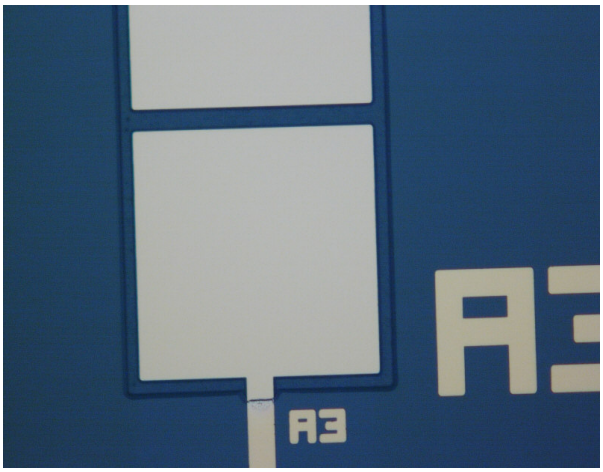


Figure 6: Close up view of an electrode pair. The black line delimits the SiO_2 opening.

IV. PROBE IMMOBILIZATION

Probes immobilization for DNA label-free detection is not an easy task in fully-electronic sensor chip. Capacitance detection for bio-sensing applications was extensively investigated since their first appearance [8]. It is based on the idea of detecting ions displacement during molecular recognition at electrodes/sample interface. A simple model may provide an intuitive explanation of this detection principle. Considering a capacitor formed by two neighboring electrodes of our chip, when a complementary DNA strand binds with the surface probes and the DNA duplex is formed, the solution ions are pushed away from the polarized metal surface. This increases the distance between the charge inside the electrodes and the ions in the electrolyte and, thus, it decreases the measured capacitance. However, the usually considered probe immobilizations have not shown sufficiently reliable properties on the measured capacitance during detection.

Evident time drift [8], very large standard deviation [9], data points largely scattered [10], poor reproducibility between electrodes [10] are always present during detection. All these phenomena are related to non-ideal capacitive behavior of the electrode/solution interface [14]. To solve this problem, a new DNA probe immobilization based on Ethylene-Glycol (EG) thiols was very recently proposed [11]. In this paper, the new probe immobilization is tested in comparison with the more traditional technique in order to verify the obtained optimization in DNA label-free detection. The two tested probe immobilizations are schematically shown in figure 7.

A. Chemicals

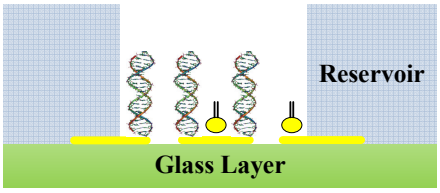
Ethylene-glycol functionalized alkanethiols differently terminated ($SH\text{-}(CH_2)_{11}\text{-}(OCH_2CH2)_3OCH_2COOH$, and $SH(CH_2)_{11}\text{-}(OCH_2CH_2)_3OH$) were purchased from Prochimia, Poland. NaCl, Na_2HPO_4, KH2PO4, KCl, H_2O2 (50%) and Absolute ethanol were purchased by Sigma, Switzerland. Different Single-stranded DNA (ssDNA) probe molecules of the same length (25-mer), amino modified, with a chain of 6 carbon atoms as a spacer and DNA target were supplied by MWG Biotech, Germany.

B. DNA Probes layer Formation

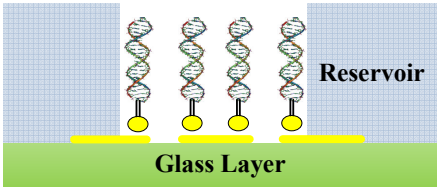
Different kinds of probe sensing layers were prepared in order to check different immobilizations. Mainly, two different immobilization techniques were tested, as shown in figure 7. In the first, SH-terminated ssDNA are directly immobilized onto chip gold electrodes and, then, 6-mercapto-1-hexanol is added as blocking agent following well established procedure [12]. In the second, Ethylene-glycol thiols monolayers are formed as probe precursors onto the electrode surface. The layers are obtained from a mixture of two differently modified alkanethiol molecules ($HS(CH_2)_{11}(OCH_2CH_2)_3OH$ and $HS(CH_2)_{11}(O\text{-}CH_2CH_2)_3O\text{-}CH_2COOH$). The mixture is prepared into a 2 mM final concentration solution of ethanol with proportion 1.96 mM of -OH terminated thiols and 0.04 mM of –COOH terminated thiols. The samples were incubated overnight, under dark conditions, in such a mixture. The samples were then rinsed and sonicated in ethanol for 10 min. Finally, NH-terminated ssDNA probes are anchored to the precursors monolayer by using well-known procedure based on N-hydroxysuccinimide (NHS) and of Ethyl-Dimethyl-aminopropyl Carbodiimide (EDC) [13]. This anchoring procedure was proposed year ago for anchoring protein probes but it was only very recently proposed for anchoring DNA probes [11].

V. CAPACITANCE MEASUREMENTS

The capacitance was acquired by using chip measurement probes station. Measurements were done by using the Charge Based Capacitance Measurements methods [11]. When measuring a capacitance with this technique, an input square wave is applied to the first electrode, while the



(A)



(B)

Figure 7: The two probe immobilizations: DNA with Mercapto-hexanol (A) and with Ethanol-glycol thiols (B).

current required to completely charge (or discharge) the capacitance is measured at the second electrode. The measured capacitance value equals the product of the average value of the output current, the applied voltage step, and the frequency of applied clock signal. In our experiments, the charging (or discharging) current was sampled in a frequency range from 80 to 100 Hz. After the ethylene-glycol monolayers formation and before the capacitance measurements, the electrode chips were left for conditioning in phosphate saline buffer (PBS) in dark for 24 hours. The conditioning was necessary to further stabilize the capacitance measurements on the so prepared electrode chips.

A. DNA Detection with Mercapto-Hexanol

DNA hybridization detection by using the SH-terminated directly immobilized onto gold of the chip electrodes and post-treated with mercapto-hexanol is shown in figure 8. Acquisitions on different chip spots are similar to that shown by the figure. In that case, the data acquired in 10 minutes on the same spot presents an evident time drift. Quite large standard deviations were registered in the DNA detection.

B. DNA detection with Ethylene-Glycol (EG)

DNA hybridization detection by using the NH-terminated immobilized onto Ethylene-Glycol Monolayers previously formed on chip gold is shown in figure 8. Acquisitions on different chip spots are similar to that showed by the figure. In that case, the data acquired in 10 minutes on the same spot do not presents time drift. Quite small standard deviations were registered during the DNA detection with this probes layer.

VI. DISCUSSION

Figures 8 and 9 compare the detection stability of DNA target surfaces obtained with the two different immobilization techniques.

The two registered capacitance values are so different because the two probe surfaces are so different. The time trend is the most important feature of the two figures. A decreasing capacitance is observed in the first graph. Similar time instability was registered in case of probes immobilized without blocking agents [9]. They are related to ion pathways that are present into the probes layer [14]. Such pathways were identified in large grooves shown by AFM images on not well-packed probes layers [11]. Similar grooves were also registered in AFM image acquired on DNA probe surfaces with mercatpo-hexanol [15]. The presence of these groves enables the gold electrode surface to be accessible to solution ions through the probes layer. This net currents flow through the DNA film accounts for time instability and interface behavior not close to that of an ideal capacitor [14]. By comparing the two figures, it is clearly evident that signal errors by using mercapto-hexanol are larger than those obtained by using ethylene-glycol precursors. We also registered similar large detection errors in the initial capacitance on the DNA probes, in case of DNA immobilized with mercapto-hexanol. Instead, the DNA detection by using Ethylene-glycol anchoring precursors is quite precise and results in quite stable capacitance signals after DNA targets hybridization, as shown by figure 9. Data related to figure 8 present only a small 0.6% of capacitance increase within the 10 minutes of the acquisition. Ethylene-glycol precursors do not present grooves into AFM images [11,15] and, thus, the DNA probes layer does not present conducting pathways to solution ions. In such a manner, an interface behavior close to that of an ideal capacitor is assured [16] and capacitance measurements are quite stable in time, as confirmed by figure 8. Another reason of this highly stable interface behavior is due to the presence of a water layer strongly coordinated by ethylene-glycol chains. This layer was envisaged by *ab-initio* calculations and confirmed by Infrared spectroscopy and QMC [11]. The strong role played by water molecules in film behavior is also confirmed by the improvement of capacitance stability after water conditioning.

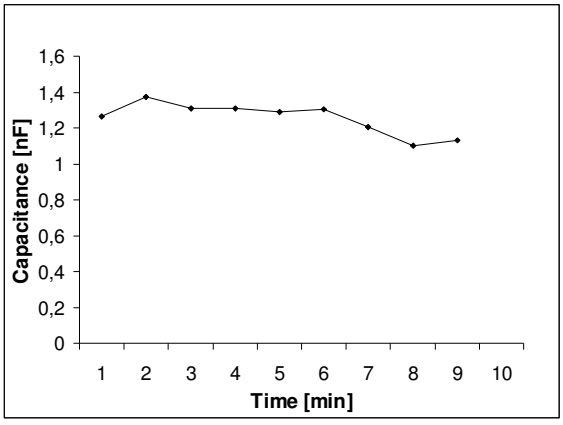


Figure 8: Capacitance measurements in time acquired on DNA hybridized on probes immobilized with Mercapto-hexanol.

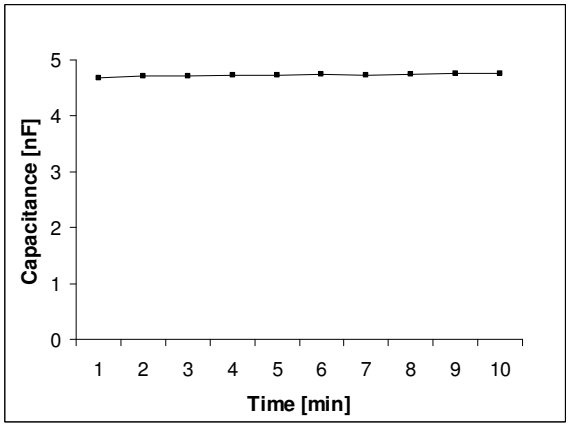


Figure 9: Capacitance measurements in time acquired on DNA hybridized on probes immobilized onto Ethylene-glycol monolayers.

VII. CONCLUSIONS

In this paper, an innovative 3D structure for label-free DNA detection with fully-electronic biochip is proposed. 3D chip architectures are considered because complex algorithms are required for organism distinguishability by using DNA detection. This leads to more intelligence on board and high level of programmability and flexibility is addressed by 3-D Chip. Disposable passive biochip fabrication was tested in order to investigate innovative replaceable bio-layers for 3D-stacks.

A novel probes immobilization was tested in order to assure reliable label-free fully-electronic detection. Future work will focus both on tests for via-based interconnections between the replaceable bio-layers and the under-posed electronics layers, and on development of analog-layer for fully-electronic capacitance detection.

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REFERENCES

[1] G.W. Klau, S. Rahmann, A. Schliep, M. Vingron, and K. Reinert, "Integer linear programming approaches for non-unique probe selection," *Discrete Appl. Math.*, 155(6-7): 840–856, 2007.

[2] C. Meneses, P. Pardalos, and M. Ragle, "A new approach to the non-unique probe selection problem," *Annals of Biomedical Engineering*, 35(4):651–658, 2007.

[3] P. Deng, M. T. Thai, Q. Ma, and W. Wu, "Efficient non-unique probes selection algorithms for DNA microarray," *BMC genomics*, 9 Suppl 1, 2008.

[4] A. Bermak, D. Martinez, "A Compact 3-D VLSI Classifier Using Bagging Threshold Network Ensembles," *IEEE Transaction on Neural Networks*, Vol. 14, No. 5, Sept. 2003.

[5] C. H. Yu, "The 3rd Dimension – More Life for Moore's Law," *International Microsystems, Packaging, Assembly* Conference (IMPACT'06), Taiwan, pp. 1-6, 2006.

[6] A. W. Topol, D. C. La Tulipe, Jr., L. Shi, D. J. Frank, K. Bernstein. S. E. Steen, A. Kumar, G. U. Singco, A. M. Young, K. W. Guarini, M. Ieong, "Three-Dimensional Integrated Circuits," *IBM J. RES. & DEV.* VOL. 50 NO. 4/5 JULY/SEPTEMBER 2006.

[7] C. Guiducci, A. Schmid, F. K. Gürkaynak, and Y. Leblebici, "Novel Front-End Circuit Architectures for Integrated Bio-Electronic Interfaces," *Design, Automation and Test in Europe (DATE '08)*, pp.1328-1333, 2008.

[8] V. M. Mirsky, M. Riepl, O. S. Wolfbeis, "Capacitive monitoring of protein immobilization and antigen-antibody reactions on monomolecular alkylthiol films on gold electrodes," *Biosensors and Bioelectronics 12* (1997) 977-989.

[9] C.Stagni, C. Guiducci, L. Benini, B. Riccò, S. Carrara, B. Samorí, C. Paulus, M. Schienle, M. Augustyniak, R. Thewes, "CMOS DNA sensor array with integrated A/D conversion based on label-free capacitance measurement," *IEEE Journal of Solid-State Circuits*, 41(2006) 2956- 2964.

[10] C. Berggren, B. Bjarnason, G. Johnansson, "An immunological Inerleukine-6 capacitive biosensor using perturbation with a potentiostatic step," *Biosensors & Bioelectronics 13*, 1061–1068, 1998.

[11] S. Carrara, V. Kumar Bhalla, C. Stagni, L. Benini, A. Ferretti, A. Cavallini, B. Riccò, B. Samorì, "New insights for using self-assembly materials to improve the detection stability in label-free DNA-chip and immunosensors, *Biosensors and Bioelectronics*, in press (2009).

[12] C. Berggren, P. Stalhandske, J. Brundell, G. Johnansson, "A feasibility study of a capacitive biosensor for direct detection of DNA hybridization, *Electroanalysis 11*, 156-160, 1999.

[13] J.Lahiri, L. Isaacs, J. T. George, M. Whitesides, "A strategy for the generation of surfaces presenting ligands for studies of binding based on an active ester as a common reactive intermediate: a surface plasmon resonance study," *Anal. Chem. 71*, 777-790, 1999.

[14] S. Carrara, F. K. Gürkaynak, C. Guiducci, C. Stagni, L. Benini, Y. Leblebici, B. Samorì, G. De Micheli, "Interface layering phenomena in capacitance detection of DNA with biochips," *Sensors & Transducers Journal*, Vol. 76 (2007), 969-977

[15] S. Carrara, A. Cavallini, Y. Leblebici, G. De Micheli, V. K. Bhalla, F. Valle, B. Samorì, L. Benini, B. Riccò, I. Vikholm-Lundin, T. Munter, "New probe immobilizations by lipoate-diethalonamine or ethylene-glycol molecules for capacitance DNA chip, submitted for publication on *IEEE Conference Proceedings of IWASA*, Bari 2009.

[16] S. Carrara, V. K. Bhalla, C. Stagni, L. Benini, B. Riccò, B. Samorì, "Improving probe immobilization for label-free capacitive detection of DNA hybridization on microfabricated gold electrodes," *Sensors and Transducers*, 88 (2008) 31-39.